Modified Antibody

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]   The present application claims priority back to US provisional application no. 60/450134, filed February 25, 2003.

FIELD OF THE INVENTION

[0002]   The present invention relates to a recombinant human antibody-based molecule called Vaccibodies, which are able to trigger both a T cell- and B cell immune response. More particularly, Vaccibodies by themselves induce such strong immune responses that adjuvants are not necessarily required. The present invention also relates to a method of treating e.g. multiple myeloma by means of the said Vaccibodies.

BACKGROUND OF THE INVENTION

[0003]   Multiple myeloma (MM) is a bone marrow cancer in which a single plasma cell clone has turned malignant and produces monoclonal immunoglobulin (Ig). MM patients have a very poor prognosis. Although high response rates and increased survival can be achieved using high dose chemotherapy followed by autologous or allogeneic stem cell grafting, the majority of patients relapse and few, if any, are cured.

[0004]   Myeloma cells produce monoclonal Ig that is unique for each tumor and thus for each individual patient. Ig is composed of two identical heavy (H) and

two identical light (L) chains. L and H chains have highly diversified variable (V) regions, VL and VH. VL and VH together form the Fv (fragment variable) that contains unique antigenic determinants called idiotopes (Id). Idiotopes collectively constitute the idiotype of the Fv (of the Ig in casu). Induction of an immune response against the idiotype, so called Id-vaccination is a promising strategy in treatment of B cell lymphomas and MM (Bendandi, Gocke et al. 1999) (Tao and Levy 1993) (Huang, Wu et al. 2004) (Hakim, Levy et al. 1996) (King, Spellerberg et al. 1998) (Biragyn, Tani et al. 1999; Biragyn, Ruffini et al. 2002), and both anti-idiotypic antibodies (Sirisinha and Eisen 1971; Hough, Eady et al. 1976) and Id-specific T cells (Lauritzsen, Weiss et al. 1994) may be of importance. However, Id is a weak self-Ag in its original context (as part of Ig). Therefore, for vaccine purposes, it is important to enhance the immunogenicity of Id.

[0005] T helper cells (CD4+ T cells) recognize their antigen (Ag) after it has been processed through engulfment of foreign proteins by APC, proteolytic breakdown into peptide fragments that are loaded onto MHC class II molecules and transported to the surface of the APC where the peptide-MHC complex is presented to T cell receptors (TCRs) of CD4 + T cells. Activated CD4+ T cells stimulate cytotoxic T cells (CD8+ T cells) and B cells with the corresponding Ag specificity, initiating a broad response against the original Ag. A major problem concerning Id-vaccination of MM patients is that Id-specific CD4+ T cells in these patients, as extrapolated from experiments in mice [18], probably are tolerant to Id V-region determinants on the highly abundant myeloma protein. MM patients who have undergone autologous stem cell transplantation (ASCT) may be in an advantageous

phase for Id-vaccination for the following two reasons: 1) relief from T cell tolerance to myeloma protein Id and 2) development of new T cells that can respond to Id-vaccination.

[0006]   Targeting of T cell epitopes to surface molecules on APC with Troybodies (Lunde, Munthe et al. 1999), which are equipped with a T cell epitope incorporated in a loop in a constant Ig domain results in increased T cell stimulation by a factor of 100-100000 (Lunde, Rasmussen et al. 2001). However, Troybodies do not include the Ag in its native conformation, such as Fv, and are therefore restricted to induction of T cell responses. Therefore, to induce an anti-Id B cell response and anti-Id Abs, it is necessary to include the complete Fv of the M component of the patient. As for induction of an anti-Id T cell response, an inclusion of the entire Fv will greatly increase the chance of including idiotope sequences binding the patient's HLA-molecules, which is a prerequisite for activation of Id-specific T cells.

[0007]   There have been several approaches for rendering idiotypes more immunogenic. Protein vaccination with complete Id+ immunoglobulins (Ig) fused with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Tao and Levy 1993), or CD40 ligand (Huang, Wu et al. 2004) enhances the level of anti-Id antibodies and results in protection against B-cell lymphoma in mice. However, scFv-GM-CSF was effective only when injected as protein and not as a DNA vaccine (Hakim, Levy et al. 1996). On the other hand, DNA vaccination employing scFv fused to IL-1β did induce tumor immunity (Hakim, Levy et al. 1996). In another approach, scFv has been genetically fused with fragment C from tetanus toxin and delivered as a DNA vaccine by intramuscular (i.m.) injection. This strategy has resulted in increased

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levels of anti-Id antibodies, Id-specific CD4+ responses and protection against lymphoma a myelomas in mice. The mechanism of adjuvant activity of tetanus toxoid fragment C is unknown (King, Spellerberg et al. 1998). In a similar approach, scFv has been fused to chemokines like MCP3, IP10 mDF2β (Biragyn, Tani et al. 1999; Biragyn, Ruffini et al. 2002) and has been used both as a DNA and as protein vaccine (Biragyn, Tani et al. 1999). In several of these studies, foreign T cell epitopes corresponding to TT fragment-C or unique fusion sequences could have contributed to responses. Heightened anti-Id antibody responses and tumor protection has been observed. The mechanism of action of scFv-chemokine is unknown. One possibility is that the chemokine moiety targets Fv to chemokine receptors on APC for enhanced delivery of scFv. Alternatively, chemokines attract APC to the site of injection. However, both the Fragment C and chemokine fusion strategies rely on monovalent binding to their target molecules (King, Spellerberg et al. 1998; Biragyn, Tani et al. 1999). This is of concern because crosslinking has been shown to be of importance for optimal stimulation of T cells, e.g. for Troybodies (Lunde, Munthe et al. 1999).

[0008]   With these considerations in mind, the inventors have designed a novel type of recombinant antibody-like molecules called Vaccibodies, a divalent molecule comprising a flexible hinge, with no FcR binding and that contain the Ag in its native conformation, with the purpose of inducing both strong Id-specific Ab and T cell responses. Vaccibodies are large and complex macromolecules, but, surprisingly, cells were able to produce and export intact molecules.

SUMMARY OF THE INVENTION

[0009]   The present invention relates to a novel type of human recombinant antibody-like molecules useful in the treatment of i.e. multiple myeloma. These molecules, called Vaccibodies, bind APC and are able to trigger both T cell and B cell immune response. Moreover, Vaccibodies bind divalently to APC to promote a more efficient initiation of an immune response. Hence, a major purpose of the present invention is to induce a strong immune response to render adjuvants redundant. Vaccibodies comprise a dimer of a monomeric unit that consists of a scFv with specificity for a surface molecule on APC, connected through a hinge region and a $C\gamma3$ domain to a scFv in the COOH-terminal end; the latter being of B cell lymphoma or myeloma origin (Fig 1), although any origin is possible due to the cassette cloning system in the expression vector. The said molecule is capable of inducing an immune response against multiple myeloma, but extension to a general vaccination strategy for any polypeptid should be feasible. The present invention also relates to a DNA sequence coding for this recombinant antibody based molecule, to expression vectors comprising these DNA sequences, cell lines comprising said expression vectors, to treatment of mammals preferentially by immunization by means of Vaccibody DNA, Vaccibody RNA, or Vaccibody protein, and finally to pharmaceuticals and a kit comprising the said molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]   The present invention will become more fully understood from the detailed description and the accompanying drawings, wherein:

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[0011] (Fig. 1) The structure of the Vaccibody. The two scFvs in white target the Vaccibody to surface molecules on APC. They may be replaced by other targeting molecules, e.g. chemokine receptors. The hinge provides flexibility of the relative orientation of the two NH2-terminal scFvs and disulfide bridges the monomers. The CH3 domains (light grey) act as a spacer between the NH_2-and COOH terminal scFvs and participate in the dimerization through hydrophobic interactions. These dimerization motifs may be replaced by other dimerization or multimerization domains. The two scFvs shown in dark grey are the antigenic moiety of the Vaccibody. These scFvs are derived from the M component, thus harboring idiotypic sequences (black). The antigenic scFv may be replaced by any polypeptide derived from an antigenic source, conferring vaccine strategies towards any antigen.

[0012] (Fig. 2) Principle. The Vaccibody is targeted to surface molecules on APC, the complex is taken up by receptor-mediated endocytosis, processed and Id-peptides are presented to CD4+ T cells on MHC class II. Simultaneously, the Id may stimulate B cells with an anti-Id BCR. These B cells will also serve as APC for the CD4+T cells. Thus, T and B cells will cooperate to enhance the response, as indicated. Anti-Id B cells will as a consequence develop into plasma cells that produce anti-Id antibodies.

[0013] (Fig.3) The MHC class II-specific Vaccibodies bind $CD19^+$ splenocytes (white). The NIP-specific Vaccibodies (negative controls - grey), do not

bind to the splenocytes. Binding was detected with streptavidinPE and 9A8bio which is a rat mAb that binds the antigenic Fv of M315 origin (VΛ1/2).

[0014]   (Fig. 4) The NIP-specific control Vaccibodies exhibits binding to the hapten NIP. Supernatant from cells transfected with genes encoding NIP-specific Vaccibodies was added to ELISA plates coated with NIP-BSA. 9A8 bio (antiVΛ1/2) was used as the secondary Ab. Similar results were obtained with a biotinylated anti IdAb (Ab2.1-4 bio).

[0015]   (Fig. 5) The Vaccibodies exhibit binding to DNP, hence the antigenic scFv is correctly folded. The M315 Ab, from which the antigenic scFv is derived, is specific for the hapten DNP. ELISA plates were coated with DNP-BSA. Supernatants from cells transfected with genes encoding various Vaccibodies were added. Binding was detected with 9A8 bio.

[0016]   (Fig. 6) APC pulsed with titrated amounts of MHC class II specific Vaccibodies stimulated polarized M315-specific T cells from TCR-transgenic SCID mice >100-1000 fold better than the NIP-specific, untargeted control Vaccibodies. There were no significant differences between the Vaccibodies with a long sequence prior to the first disulfide bridge in the hinge (h1+h4) compared to the Vaccibodies with a short hinge sequence above the first disulfide bridge (h4).

[0017] (Fig. 7) The MHC class II-specific Vaccibodies induce a strong anti-Id Ab response in the absence of adjuvant. BALB/c mice were injected with Vaccibodies, 20μg and 200μg, respectively. Blood samples were taken on different time points for sera analysis. Shown is data from sera taken on day 28, 14 days after the second immunization of Vaccibodies. The MHC class II-specific Vaccibodies induced a strong anti-Id Ab response. The Vaccibodies with the longest hinge (h1+h4) induced the strongest anti-Id Ab response reaching 3-4μg/ml in sera.

[0018] (Fig. 8) Construction of the two hinge-Cγ3 variants derived from hIgG3 by PCR. The templates were from pUC19 containing modified hIgG3 constant regions were the h4 exon were connected to the CH3 domain (A) or the h1 exon were connected to the h4 exon further connected to the CH3 domain (B) (Olafsen T et al, 1998). The primers inserted HindIII (5') and SfiI (3') restriction enzyme sites. The hinge and CH3 domain are connected by a triplicate of the amino acids GlyGlyGlySerSer.

[0019] (Fig. 9) Construction of the hinge-Cγ3 segments derived from mIgG2b. The hinge and the CH3 genes were amplified from a pUC18 vector containing the constant region of mIgG2b by PCR with two primers encoding a HindIII (5') and a SfiI (3') restriction enzyme site. The two PCR fragments were joined by PCR SOEing. In this reaction, the hinge and CH3 domain were connected by a triplication of the amino acids Gly-Gly-Gly-Ser-Ser.

[0020] (Fig. 10) Construction of the scFv derived from the myeloma protein M315. The cDNA that functioned as a template in the PCR reactions were derived from mRNA extracted from MOPC315.4 cells. The V regions were joined by PCR SOEing resulting in a scFv. In this reaction, the V regions were connected by a triplicate of GlyGlyGlyGlySer. Furthermore, the gene fragments encoding the complete scFv were flanked by Sfil and Sall restriction enzyme sites.

[0021] (Fig. 11) Joining of the hinge-Cγ3 segments and the M315 scFv by PCR SOEing. This reaction introduced the Sfil site 5' of the antigenic scFv encoding region.

[0022] (Fig. 12) Subcloning of the hinge-Cγ3-M315 scFv into pUC19. Three different dimerization motifs were included, derived from mIgG2b or IgG3. In all cases, they consisted of hinge followed by a triplicate of GlyGlyGlySerSer and CH3. Two different hinges were derived from hIgG3, one consisting of h1 linked to h4, and one consisting of h4, only.

[0023] (Fig. 13) Removal of two inconvenient BamHI restriction enzyme sites within the gene fragment encoding the antigenic scFv by QuickChange PCR.

[0024] (Fig.14) Introduction of stop codon, a Sfil and a BamHI restriction enzyme site downstream of the coding region by QuickChange PCR.

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[0025] (Fig.15) Subcloning into the C cassette of the expression vector pLNOH$_2$ on HindIII-BamHI.

[0026] (Fig.16) Cloning of the V regions specific for NIP and MHCII. The V regions were amplified and joined by PCR soeing resulting in scFvs. The linker connecting the V regions consists of a triplicate of GlyGlyGlyGlySer. The gene fragments encoding the complete scFvs are flanked by BsmI/MunI and BsiWI sites. Linkers and restriction sites were introduced in the PCR reactions.

[0027] (Fig.17) Subcloning into the expression vector pLNOH2 on BsmI/MunI and BsiWI.

[0028] (Fig.18) The final Vaccibody construct.

[0029] (Fig. 19) Detailed figure of Vaccibody gene construct. The targeting unit is inserted between the BsmI/MfeI and BsiWI restriction enzyme sites (The V cassette of the pLNOH$_2$ vector). The hinge-Cγ3-Fv315 is inserted between the HindIII and BamHI sites into the C cassette of pLNOH2. The hinge and the Cγ3 domain as well as the two scFv's are connected with (G$_4$S)$_3$ linkers (black boxes). The Cγ3 and the Fv^{315} are connected through a GLSGL linker. The Fv^{315} is inserted between two nonidentical SfiI restriction enzyme sites. The antigenic unit and dimerization motif may be of any origin appropriate. Also, functional fragments of

Cγ3 may be employed, or a sequence which is substantially homologous to the Cγ3 sequence or Cγ3 fragments.

[0030] (Fig. 20) Vaccibodies are secreted as functional molecules. Two distinct Vaccibodies were tested, one carrying the hapten specific Fv^{NIP} as targeting unit, while the other carried the MHC class II-specific Fv^{I-E} as targeting unit. Both carried the scFv from M315 (Fv^{315}) as antigenic unit. a) 10% SDS-PAGE of metabolically labeled Vaccibodies immunoprecipitated from culture supernatants of transfectants with or without reduction of disulfide bonds by mercaptoethanol (ME). b) DNP-specificity of the Vaccibodies was measured by ELISA. Supernatants from NSO cells transfected with Vaccibodies were added to ELISA plates coated with DNP-BSA. Data are illustrated as mean of triplicates and error bars indicate SEM. c) NIP-specificity was measured by ELISA. ELISA plates were coated with NIP-BSA. Vaccibodies in both b) and c) were detected by either 9A8-bio (αVλ1/2) or Ab2.1-4 (specific for Id of Fv^{315}) Fv^{315} carries Vλ2 and FvNIP carries Vλ1 and will both bind 9A8 mAb. Only Fv^{315} will bind Ab2.1-4 mAb. Fv^{I-E} carries Vκ and will bind neither of the mAbs.

[0031] (Fig. 21) Production of Vaccibodies by intramucular injection of naked DNA plasmids followed by *in vivo* electroporation. Serum samples were collected on day 14. Vaccibody plasmids were injected into I-E^d positive BALB/c mice, which were subsequently electroporated. a) Level of Vaccibodies in sera was measured by ELISA, with DNP-BSA and 9A8bio as described previously. b) The

same day 14 sera samples were analyzed for anti-Id antibodies by ELISA. Microtiter plates were coated with M315 and 187-bio (anti-mouse κ Ab) was used for detection. c) Comparison of detectable Vaccibody levels and anti-Id antibodies. The amount of detectable Vaccibodies in sera is shown on the y-axis and the level of αId-Abs is shown on the x-axis.

[0032] (Fig. 22) Tumor avoidance. BALB/c mice were immunized once with naked plasmids encoding MHC class II specific Vaccibodies (Fv^{I-E} Fv^{315}), nontargeted NIP-specific Vaccibodies (Fv^{NIP} Fv^{315}) or 0.9% NaCl by i.m. immunization into the two quadriceps muscles (25μg/muscle) followed by *in vivo* electroporation. They were challenged with 1.6×10^5 MOPC315.4 myeloma cells s.c. and the first day tumor take were recorded. A tumor of >3 mm was scored as positive tumor take.

[0033] (Fig. 23) Induction of protective immunity against the MOPC315.4 plasmacytoma. BALB/c mice were immunized once with naked plasmids encoding MHC class II specific Vaccibodies (Fv^{I-E} Fv^{315}), nontargeted NIP-specific Vaccibodies (Fv^{NIP} Fv^{315}) or 0.9% NaCl by i.m. immunization into the two quadriceps muscles (25μg/muscle) followed by *in vivo* electroporation. They were challenged with 1.6x105 MOPC315.4 myeloma cells s.c. and their survival were compared.

[0034] (Fig. 24) Level of M315 myeloma protein in sera of mice on a) day 18 and b) day 24 after MOPC315.4 challenge in BALB/c mice vaccinated i.m. with

Vaccibody plasmids followed by *in vivo* electroporation. M315 in sera samples were measured by ELISA coated with anti-Id-mAb (Ab2.1-4) and detected by biotinylated anti-IgA mAb (8D2).

[0035] (Fig. 25) Chemokine Vaccibodies are secreted as functional molecules. MIP-1α Fv^{315} has mouse macrophage inflammatory protein 1 α as the targeting unit and scFv from M315 ($Fv^{315)}$ as the antigenic unit. Functionality of MIP-1α in Vaccibody format was measured in ELISA. Supernatants from 293E cells transfected with Vaccibodies were added to ELISA plates coated with anti-mouse MIP-1α mAb (R&D Systems) and detected by 9A8-bio (αVΛ1/2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0036] The following description of the preferred embodiment(s) is merely exemplary in nature and is in no way intended to limit the invention, its application, or uses.

[0037] The present invention relates to a recombinant human antibody-based molecule, called Vaccibodies, comprising dimers of a monomeric unit that consist of a single chain fragment variable (scFv) of immunoglobulins (Ig) with specificity for surface molecules on Ag presenting cells (APC), connected through a hinge region and a Cγ3 domain to a scFv in the COOH-terminal end, the latter being derived from a myeloma protein (Fig 1), although any origin is possible due to the cassette cloning system of the expression vector. The hinge region and the Cγ3 domains (carboxyterminal C domain of Ig) contribute to dimerization of the

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Vaccibody through disulfide bridges in the hinge and strong hydrophobic interactions between the two $C\gamma3$ domains. Hence, the dimeric product will include two preferably identical scFvs with binding specificity for the same surface molecules on APC (Fig 2), enabling bivalent binding. Bivalent binding (i.e. crosslinking) is of importance to trigger activation of the target cell and thereby initiation of an immune response. Also, bivalent binding provides increased binding strength due to avidity effects, and increases the likelihood of receptor mediated endocytosis into the APC and subsequent degradation inside the APC. Furthermore, the bivalent binding may provide important receptor mediated intracellular signaling to the APC. The scFvs with a targeting function are either derived from B cell hybridomas expressing monoclonal antibodies (mAbs) that bind to surface molecules on APC, or they may be derived from any source, e.g. phage display libraries. The use of scFvs from B cell hybridomas as the targeting moiety opens for a great range of possible targets due to the large collection of B cell hybridomas that produce mAbs which bind different surface molecules on APC. Furthermore, one may choose the nature of the signal given to the targeted cell by employing agonistic or antagonistic mAbs. Growing knowledge of Ab-Ag interactions will allow the improvement of the binding affinity of such mAbs to their Ag by amino acid replacements in the binding sites. This can be performed by ordinary site-directed mutagenesis. For vaccine purposes, an attractive approach is to target the Vaccibodies to surface molecules expressed exclusively on subsets of dendritic cells (DC) that are able to initiate a strong, specific immune response towards the patients own Id. Examples of such target surface molecules on APC are CD40, Toll-like receptors and chemokine receptors.

Because the targeting scFv is inserted into the V cassette of the expression vector pLNOH2 (Norderhaug, Olafsen et al. 1997), it is easily exchanged with other scFvs (Fig 17).

[0038]   The crucial dimerization motifs in the Vaccibodies constructed in the examples so far, include hinge regions and Cγ3 domains. The hinge contributes to the dimerization through the formation of interchain disulfide bridges. In addition, it functions as a flexible spacer between the domains allowing the two scFvs with targeting tasks to bind simultaneously to two target molecules expressed with variable distances (Fig 2). The C□3 domains contribute to the dimerization through hydrophobic interactions. These dimerization motifs can be exchanged with other multimerization moieties (e.g. from other Ig isotypes/subclasses).

[0039]   The C-terminal scFv derived from the monoclonal Ig produced by myeloma or lymphoma cells, also called the myeloma/lymphoma M component, can be genetically exchanged with other scFvs or any antigen because the vector has been constructed with a Sfi I restriction site (Fig 8). Therefore, the scFv derived from the model myeloma protein used in the present example is easily swapped with scFv from the M component of any patient with B cell lymphoma or multiple myeloma. Thus, this vector will allow for rapid construction of individual patient specific vaccines. The dimeric structure of the Vaccibody not only affords crosslinking, but should also allow double loading of the patient Fv to the APC per Vaccibody molecule compared to a single scFv combined to a single targeting moiety. Furthermore, there is in a single Vaccibody duplication of serological idiotypic determinants, which might be of importance for the anti-Id B cell response. The

Vaccibodies lack a CH2 domain and hence all FcR binding sites, and should therefore exclusively be taken up through its target molecules, such as MHC class II in the example used and not by any FcRs, ensuring that a large proportion of the vaccine will arrive at the intended target cells. This is in contrast to vaccines that exert their effect through the binding to an FcR on a target cell (Ravetch and Bolland 2001).

[0040]   Vaccibodies can be extended to a general medical treatment through induction of an immune response against any polypeptide of any origin. It is possible to replace the idiotypic scFv with other antigenic sequences of sufficient length to allow proper folding of the polypeptide. This sequence may be derived from other cancer proteins or infectious agents. Insertion of such a sequence in a Vaccibody format might also lead to activation of both arms of the immune response, similar to the Vaccibodies that are described herein, which comprise the idiotypic scFv. Immunization by means of Vaccibody protein, Vaccibody DNA, or Vaccibody RNA, the latter two executed e.g. by intramuscular injection followed by electroporation (See Examples), are all feasible methods.

[0041]   The scFvs on the NH2- terminal end of the Vaccibodies target the Vaccibodies to APC through binding to surface molecules (Fig 2), and in the example shown they bind to MHC class II. MHC class II is expressed on all professional APC, so the Vaccibodies described herein are able to target B cells, DC and macrophages. Targeting of conventional Ag-Ab complexes to MHC class II induces activation of specific CD4+ T cells (Snider and Segal 1987; Casten and Pierce 1988) . Targeting of Troybodies to MHC class II has previously been shown

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to enhance Ag presentation and T cell activation in vitro as well as in vivo (Lunde, Western et al. 2002). In the antigenic region of the molecule, the Vaccibodies of the examples contain the scFv of the myeloma protein M315 derived from the BALB/c plasmacytoma MOPC315.4. The λ2315 light chain of M315 harbors three defined somatic mutations in the CDR3 loop and functions as a model idiotypic T cell epitope in a well defined system (Bogen, Malissen et al. 1986; Bogen and Lambris 1989).

[0042]   The Vaccibodies have been genetically assembled, and the DNA transfected into NSO cells, 293E cells and Cos-7 cells. Transfectants produce and secrete the recombinant Vaccibody molecules. The targeting scFvs at one end of the Vaccibodies exhibit binding to MHC class II (Fig 3). The antigenic scFv at the other end of the Vaccibody binds DNP (di-nitro-phenyl, the specificity of M315) (Fig 5). Thus, both the targeting and antigenic scFvs retain the same folding pattern as in their original context. We have evidence that Vaccibodies have the ability to induce strong T cell responses through their binding to APC and presentation of Id-peptides on class II molecules to Id-specific CD4+ T cells in vitro (Fig 6). Furthermore, since they have intact Fv of the M-component, they elicit anti-Id antibodies in significant amounts in vivo when injected into BALB/c mice without adjuvants (Fig 7).

[0043]   To determine if MM patients treated with ASCT achieve remission with a low serum myeloma protein concentration, ELISA should performed for each patient's myeloma protein because routine assays (agarose gel electrophoresis, combined with immunofixation) have only a sensitivity of about 0.2-1 mg/ml, which is far too insensitive. The kinetic data of the serum myeloma protein levels will indicate if and when Id-vaccination may best be performed post ASCT to avoid the problem

of T cell tolerance of newly educated thymic emigrants. To achieve this, mice are immunized with DNA encoding patient specific Vaccibodies by in vivo electroporation of muscle cells. Sera from immunized mice are absorbed on anti human Ig-Sepharose to remove crossreactive antibodies and thereafter eluted to obtain purified highly Id-specific antibodies. Sandwich ELISAs specific for each patient's myeloma are performed as follows: The purified anti-Id Ab from mice is coated in wells. Serum from the patient in casu is added. Myeloma protein binding to anti-Id antibodies will be detected by use of Ab specific for human IgG or IgA. The sensitivity of such sandwich ELISAs is usually < 1 ng/ml, which is > 106 times more sensitive than routine assays. Furthermore, to monitor development of new T cells, profile of T cells in blood will be monitored by flow cytometry with Vβ-specific mAbs, in combination with other markers.

[0044]   The present invention relates to a pharmaceutical comprising the above described recombinant based antibody, DNA/RNA sequences, or expression vectors according to the invention. Where appropriate, this pharmaceutical additionally comprises a pharmaceutically compatible carrier. Suitable carriers and the formulation of such pharmaceuticals are known to a person skilled in the art. Suitable carriers are e.g. phosphate-buffered common salt solutions, water, emulsions, e.g. oil/water emulsions, wetting agents, sterile solutions etc. The pharmaceuticals may be administered orally or parenterally. The methods of parenteral administration comprise the topical, intra-arterial, intramuscular, subcutaneous, intramedullary, intrathekal, intraventricular, intravenous, intraperitoneal or intranasal administration. The suitable dose is determined by the

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attending physician and depends on different factors, e.g. the patient's age, sex and weight, the kind of administration etc. The present invention also relates to a kit comprising Vaccibody DNA, RNA, or protein for diagnostic, medical or scientific purposes.

[0045] The above described nucleotide sequences may preferably be inserted into a vector suited for gene therapy, e.g. under the control of a specific promoter, and introduced into the cells. In a preferred embodiment the vector comprising said DNA sequence is a virus, e.g an adenovirus, vaccinia virus or an adeno-associated virus. Retroviruses are particularly preferred. Examples of suitable retroviruses are e.g. MoMuLV or HaMuSV. For the purpose of gene therapy, the DNA/RNA sequences according to the invention can also be transported to the target cells in the form of colloidal dispersions. They comprise e.g. liposomes or lipoplexes.

[0046] In a preferred embodiment of the present invention naked Vaccibody DNA construct is injected intra-muscularly into mice, whereupon the site of injection is subject to in vivo electroporation. This DNA vaccination resulted in production of Vaccibody protein which conferred life-saving protective immunity on a majority of the mice.

MATERIALS AND METHODS

Mice

[0047]   BALB/cABom were from Bomholtgaard (Ry, Denmark). The λ2315-specific TCR-transgenic mice on a BALB/c background (Bogen, Gleditsch et al. 1992)were bred in our animal facility.

Cell lines

[0048]   The 14-4-4S Hybridoma (Ozato, Mayer et al. 1980) and NSO cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). 293E cells, a variant of the 293 cell line expressing the Epstein-Barr virus EBNA1 protein.

Construction of Vaccibodies

[0049]   The gene for the hIgG3 hinge and CH3 domain was cloned from the pUC19 vector containing hinge genetically combined with Cγ3 genes of the hIgG3 subclass (Olafsen, Rasmussen et al. 1998). Two variants of the hinge length in the humanized Vaccibodies were made; one with just the h4 exon connected to the CH3 domain (sh) and one with both exon h1 and h4 connected to the CH3 domain (lh) (Fig 8). The primers included restriction enzyme sites (underlined): 5'h4: <u>tag caa gct</u> tgg cca gcg cag gga g; 3'CH3: ca<u>g gcc acc gag gcc</u> ttt acc cgg aga cag gga. The h1 exon were introduced directly upstream of the h4 exon by QuickChange PCR using these primers Qh1a: ctccaatcttctctctgca **gag ctc aaa acc cca ctt ggt**

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gac aca act cac aca gag ccc aaa tct tgt gac ac and Qh1b: gt gtc aca aga ttt ggg ctc

tgt gtg agt tgt gtc acc aag tgg ggt ttt gag ctc tgcagagagaagattgggag.

[0050] The murine Vaccibodies have a complete hinge and CH3 domain of

the mIgG2b subclass picked up by PCR from a pUC18 vector containing the Cγ2b

genes (Fig 9). The primers included restriction enzyme sites (underlined) or linkers

(bold) with the complementary sequences (italic): 5'hinge: tagc<u>aagctt</u> ca gag ccc

agc ggg ccc; 3'hinge: 5'**tcc acc tcc gct gct tcc acc gcc** tgg gca ttt gtg aca ctc ctt g;

5'CH3: **gga agc agc gga ggt gga agt gga** ggg cta gtc aga gct cca ca; 3'CH3: ca<u>g</u>

<u>gcc acc gag gcc</u> acc cgg aga ccg gga gat g. The hinge and the CH3 domain were

then joined by PCR SOEing.

[0051] The Antigenic V region genes were cloned from the plasmacytoma

MOPC315.4 (Eisen, Simms et al. 1968). The V regions were obtained by extracting

mRNA from the MOPC315.4 cell line with oligo (dT)-coated magnetic Dynabeads

(Dynal). First strand cDNA were then made and used as template for PCR

amplification of the V region genes using specific primers annealing to the exact

ends of the M315 V region sequences. The primers included restriction enzyme sites

(underlined) or linkers (bold) with the complementary sequences (italic). The primer

sequences were: 5'VH: <u>ggc ctc ggt ggc c</u>tg gat gta cag ctt cag gag tca; 3'VH: **gcc**

aga gcc acc tcc gcc aga tcc gcc tcc acc tga gga gac tgt gag agt ggt; 5'VL: **ggc**

gga ggt ggc tct ggc ggt ggc gga tcg cag gct gtt gtg act cag gaa; 3'VL: gac<u>g tcgac</u>

tag gac agt gac ctt ggt tcc. The VH and VL genes were then joined by PCR soeing to

a scFv format (Fig 10).

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[0052]   The complementary sequences in the tags 3' of the Cγ3 coding region and 5' of the M315 VH coding region enabled the M315 scFv to be combined with the three different hinge-CH3 genes by PCR SOEing (Fig 11). The products of this reaction were then digested with HindIII and SalI and subcloned into a pUC19 vector (Fig 12). Two BamHI restriction enzyme sites inside the V regions of M315 were removed by QuickChange PCR (Fig 13) using primers: BamHI VL1:at gcc aac tgg ata caa gaa aaa cc; BamHI VL2: gg ttt ttc ttg tat cca gtt ggc at, BamHI VH1: tgg aac tgg ata cgg cag ttt cc and BamHI VH2: gg aaa ctg ccg tat cca gtt cca. A following QuickChange PCR using primers: 3'VL stop1: gtc act gtc cta tga ggcctgcagggcc ggatcc gtcgactctag and 3'VL stop2: cta gag tcg ac ggatcc ggccctgcaggcc **tca** tag gac agt gac, were then performed to introduce a stop codon (bold), a SfiI and a BamHI restriction enzyme site (underlined) downstream of the coding region (Fig 14).

[0053]   The final construct is then digested with HindIII and BamHI and subcloned into the expression vector pLNOH$_2$ (Fig 15) (Norderhaug, Olafsen et al. 1997).

[0054]   The V region genes providing specificity for MHC class II had previously been cloned from the 14-4-4S hybridoma (Lunde, Western et al. 2002), which produces an Ab specific for the Eα chain (determinant Ia.7) of the I-E MHC class II molecule (Ozato, Mayer et al. 1980). Specific primers annealing to the exact

ends of the V region sequences with tags designed to include restriction enzyme sites (underlined) or linker sequences (bold) with the complementay sequences (italic). The primer sequences were: 5'VL: gac att caattg aca cag tct tct cct gct tcc; 3'VL: **gcc aga gcc acc tcc gcc aga tcc gcc tcc acc** ttt gat ttc cag ctt ggt gcc; 5'VH: **ggc gga ggt ggc tct ggc ggt ggc gga tcg** cag gtc cag ctg cag cag t; 3'VH: ga cgtacg actcacc tga gga gac ggt gac tga gg. The V region genes giving specificity for the hapten NIP (Neuberger 1983) were designed with the similar tag sequences except for the 5'VL primer: 5'VL: ggtg tgcattcc cag gct gtt gtg act cag gaa; 3'VL: **gcc aga gcc acc tcc gcc aga tcc gcc tcc acc** tag gac agt cag ttt ggt acc t; 5'VH: **ggc gga ggt ggc tct ggc ggt ggc gga tcg** cag gtc caa ctg cag cag cc; 3'VH: ga cgtacg a ctc acc tga gga gac tgt gag agt ggt. The VL and VH were then joined by PCR SOEing (Fig 16) and subcloned into the V cassette pLNOH2 vector containing the hinge-CH3-scFvM315 genes (Fig 17 and Fig 18). Likewise, other V genes conferring a desired specificity are isolated from hybridomas or from phage selected from phage display libraries. They are then PCR amplified using primers designed in the same manner as above and subcloned after PCR SOEing in the targeting-cassette (Fig. 17 and 18). Rearranged VH and VK genes conferring specificity for HLA-DP were PCR amplified from cDNA from the 22C1 hybridoma, which produces an antibody with pan HLA-DP specificity. The V genes were reamplified with new primers containing sites for direct cloning into the expression vectors pLNOK and pLNOH2 (Norderhaug and Olafsen, 1997); 5'-VL, ggtgtgcattccgacattgtgctcacc; 3'-VL, cgtacgttctactcacgttttatttccagct; 5'-VH, gtgcattccgaggtgcagctgcaggagtct; 3'-VH,

cgtacgactcacctgaggagaccgtagc. Furthermore, scFV was generated by PCR SOEing using the following primers:

> 5'VL, g gtg <u>tgcattc</u> cga cat tgt gct cac c
>
> 3'VL: **gcc aga gcc acc tcc gcc aga tcc gcc tcc acc** gtt tta ttt cca gct
>
> 5'VH: **ggc gga ggt ggc tct ggc ggt ggc gga tcg** gag gtg cag ctg cag
>
> gag tct
>
> 3'VH, <u>cgtacg</u> act cac ctg agg aga ccg tag c

[0055]   In 3'VL and 5'VH the sequences in bold+italics are complementary and antiparallell, thus hybridising to generate the gene fragment encoding the linker region. Anti CD14 V regions are cloned from the mouse hybridoma 3C10 (ATCC).

[0056]   The chemokine genes were cloned from thioglycolate stimulated peritoneal macrophages. 4ml 2% thioglycolate were injected i.p. into Balb/c mice. 3 days later peritoneal macrophages were collected and mRNA was extracted with oligo (dT)-coated magnetic Dynabeads. First strand cDNA was made and used as template for PCR amplification of chemokine genes (RANTES and MIP-1α) using specific primers: 5'MIP-1α: ggtg <u>tgcattc</u> cgc gcc ata tgg agc tga cac, 3'MIP-1α: ga <u>cgtacg</u> act cac ctg cat tca gtt cca ggt cag tg 5'RANTES: ggtg <u>tgcattc</u> c gcc tca cca tat ggc tcg g 3'RANTES: ga <u>cgtacg</u> a ctc acc tga cat ctc caa ata gtt gat gta ttc. The different targeting unit genes were then digested with MunI and BsiWI or BsmI and BsiWI, respectively and subcloned into the V cassette pLNOH₂ vector containing the hinge-CH3-scFvM315 genes (Fig 17 and Fig 18). CD40 ligand is cloned from T cells

that are activated with LPS for 4 hours before mRNA is extracted for preparation of cDNA. The cDNA is used as template in a PCR reaction with primers specific for the CD40 ligand sequence. Furthermore, this sequence is reamplified with primers designed to facilitate subcloning in the targeting cassette as described above.

Production and purification of Vaccibodies

[0057]   The pLNOH$_2$ vector carrying the Vaccibody genes was transfected into NSO cells by electroporation, and supernatants from single colonies resistant to 800µg/ml G418 were analyzed for Vaccibody secretion after 2-3 weeks, using ELISA. DNP-BSA was used as coat, and biotinylated rat-anti mouse VΛ1/2 (9A8-bio) was used for detection. The NIP-specific Vaccibodies were additionally screened in an ELISA using NIP-BSA as coat. The cells selected for high Vaccibody production were grown in Rollerbottles (VWR) and affinity purified from supernatant using a column made by DNP-lysine (Sigma) coupled to fast flow Sepharose. The Vaccibodies were eluted with 0.05M DNP-glycine (Sigma) and the flow-through was run on an ion-exchange Cl- Dowex 1x8 resin column (Sigma). The eluted Vaccibodies were dialyzed against PBS/0.05%NaN3 and sterile PBS, before the vaccibody concentration were calculated from absorbance values at 280nm.

Ab and flow cytometry

[0058]   Ab and reagents used for flow cytometry were 9A8 biotin, FGK.45 biotin, streptavidin PerCP, anti-CD19 PE and anti-mIgG2a PE (BD Pharmingen). BALB/c spleen cells were double stained with anti-CD19 PE and Vaccibodies.

Bound Vaccibodies were detected by 9A8-bio and streptavidin PerCP. Twenty thousand cells were run on FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using the WinMDI software.

Metabolic labelling and immunoprecipitation

[0059] $2x10^6$ cells were labelled for 6h at 37°C in RPMI lacking methionine, cysteine (BioWhittaker) containing 100μCi ^{35}[S]-methionine, cysteine (Amersham). The SN was harvested and immunoprecipitated with rat anti-mouse VΛ1/2 (9A8) on a wheel ON at 4°C. 10μl Dynabeads coated with sheep anti-rat IgG (Dynal AS, Oslo, Norway) were incubated with the precipitate for 1h on a wheel and the Dynabeads were collected with a Dynal Magnetic Particle Concentrator rack (Dynal MPC). The beads were washed three times in ice cold PBS with 1%NP40 and resuspended in 10μl 1x sample buffer. The proteins were eluted from the beads by incubating the samples at 95°C for 3 minutes. The Vaccibodies were run on a 10% SDS-PAGE gel, with a 5% stacking gel, at 40mA for 1h, using a BIO RAD miniprotean II gel electrophoresis apparatus. The gels were subsequently fixed in 30% methanol and 10% acetic acid for 30 minutes prior to 30 min incubation with Amplify (Amersham), before drying and exposing to BIOMAX-MR film (Eastman Kodak Company, Utah, USA).

T cell proliferation assay

[0060]   Irradiated (2000rad) BALB/c splenocytes ($5x10^5$cells/well) were used as a source of APC. Titrated amounts of different MHC CLASS II- and NIP-

specific Vaccibodies were added to the splenocytes. A 91-107 $\lambda 2^{315}$ synthetic peptide were used as a positive control. The assays were put up in 150μl cultures in 96-well flat-bottom microtiter wells and incubated for 4 h at 37°C (Lunde, Western et al. 2002). The cultures were then washed three times before addition of 200μl polarized $\lambda 2^{315}$-specific Th2 cells (2x10^4) derived from TCR transgenic SCID mice. After 48h, the cultures were pulsed for 16-24h with 1μCi ^{3}[H] dThd. The cultures were harvested and, and incorporated ^{3}[H] dThd was measured using a TopCount NXT scintillation counter (Packard, Meriden, CT).

In vivo experiments

[0061] BALB/c mice were injected subcutaneously (s.c) with 200μg or 20μg purified Vaccibody proteins in PBS on day 0, 14 and 28. Blood samples were taken on day 14 and 28 before revaccination and then on day 35, 42 and 49, before they were sacrificed according to the Humane End Point procedure.

Measurement of antibody responses

[0062] Anti-idiotypic Abs against M315 were measured by ELISA. The wells were coated with 2μg/ml M315. Anti-Id Ab in the sera were detected by a biotinylated anti-mouse Vκ Ab (187.1 bio), anti-mouse IgG1 bio or anti-mouse IgG2a bio (both from BD Pharmingen). Ab2.1-4 (an anti-Id mAb that bind $\lambda 2^{315}$) was used as standard.

Vaccination

[0063] Protein vaccination: BALB/c mice were injected subcutaneously (s.c) in the right flank region with 20μg or 200μg purified class II– or NIP specific Vaccibodies (F_v^{I-E} F_v^{315}, F_v^{NIP} F_v^{315}) in PBS on day 0 and 14. PBS was injected as negative control. Blood samples were collected from the leg vein on different time points after the last immunization. Anti-idiotypic antibodies with specificity with specificity for F_v^{315} were measured by ELISA. The wells were coated with 2μg/ml M315. Anti-Id Ab in the sera were detected by a biotinylated anti-mouse κ mAb (187.1 bio). Ab2.1-4 (an anti-Id mAb binding F_v^{315} (Kristoffersen, Hannestad et al. 1987) was used as standard.

DNA vaccination and electroporation

[0064] Five to ten weeks old Balb/c mice were purchased from Bomholtgaard (Ry, Denmark). The animals were anaesthetized by intraperitonal injection with 9 g Pentobarbital/mice and the legs were shaved. Conductive gel was applied at the skin and 50 μl vector DNA diluted in 0.9% NaCl, was injected into the quadriceps. Following injection, electroporation was performed, by applying rod electrodes to the skin near the site of the injection and subjecting the site to an electrical potential comprising 10 trains of 1000 pulses each, with a pulse length at two times 200 Sec (positive 200Sec and negative 200 Sec) with 600 s interval between each pulse and with a current limit of 50 mA (about 150-174 V/cm) (Tollefsen, Tjelle et al. 2002).

[0065]   Blood samples were collected from the leg vein on different time points and heart puncture was performed on the day they were sacrificed. Serum samples were analyzed for the presence of correctly folded Vaccibodies. The ELISA was performed with DNP-BSA as coat and 9A8-bio as detected Ab, as described above. In addition, serum samples were analyzed for anti-Id Abs by ELISA as described above.

Tumor challenge

[0066]   Protein Vaccibodies-MOPC315.4: BALB/c mice (6-10 weeks old) were injected s.c. with 160 μg class II-or NIP-specific Vaccibodies in PBS in the right flank region on day 0 and 14. On day 28, 1.6×10^5 MOPC315.4 cells were injected s.c. on the right flank. Mice were inspected twice weekly. Tumor size development was monitored by palpation and use of a caliper. A tumor of 3 mm in diameter was scored as tumor take. Mice were killed when tumor size reached 20 mm with no sign of tumor necrosis.

[0067]   DNA Vaccibodies-MOPC315.4: DNA vaccination was performed at day 0 as described above. On day 14, 1.6×10^5 MOPC315.4 cells were injected s.c. in the right flank region. Tumor size development was monitored by palpation and use of a caliper. The mice were sacrificed when the tumor size reached 20 mm. Blood samples were collected on different time points from the leg vein. Levels of M315 myeloma protein in sera were quantified in a sandwich ELISA with Ab2.1-4 as coat by biotinylated anti-Cα (8D2) mAb as detection Ab. Tumor size, tumor take,

survival curves and statistical analyses were calculated by use of Graph Pad Prism 3.0 software (San Diego, CA).

EXAMPLES

[0068]   By way of example the following experiments demonstrate that Vaccibodies bind APC and are able to trigger both T cell and B cell immune response. Moreover, the following experiments show that Vaccibodies induce a strong immune response rendering adjuvants redundant. The experiments demonstrate that said molecule is capable of inducing an immune response against multiple myeloma and, further, the feasibility of treatment of mammals by immunization by means of Vaccibody DNA or Vaccibody protein. The experiments also demonstrate that another attractive approach is to target the Vaccibodies to surface molecules expressed exclusively on subsets of dendritic cells (DC), like e.g. chemokine receptors. The following examples are meant to illustrate how to make and use the invention. They are not intended to limit the scope of the invention in any manner or to any degree.

Example 1

[0069]   Vaccibodies are produced and secreted as functional dimerized molecules and is itself bound by the anti-Vλ1/2 antibody (9A8) (Bogen 1989) and the anti-idiotypic antibody Ab2.1-4 (Lauritzsen, Weiss et al. 1994).

[0070] The M315 mAb binds the hapten di-nitro-phenyl (DNP) (Eisen, Simms et al. 1968). Therefore, to verify that Vaccibodies were produced, secreted and correctly folded as functional molecules, the antigenic units of Vaccibodies were tested in ELISA for their capability to bind DNP, 9A8 and Ab2.1-4 mAbs. Fig 20b shows that both the NIP-specific and the MHCII-specific Vaccibodies, that both have scFv^{315}, bind DNP, 9A8 and Ab2.1-4. This was the case with all Vaccibodies containing scFv^{315}, both the ones with long human dimerization unit, short human dimerization unit and murine dimerization unit (Fig 5). We next tested the targeting units of the Vaccibodies. These were found to be correct; first, the NIP-specific Vaccibodies bound NIP-BSA in ELISA (Fig 4 and Fig 20c), while the MHCII-specific Vaccibodies did not (Fig 20c). Second, the MHCII-specific Vaccibodies bound to I-E expressing BALB/c splenic B cells (H-2^d) as detected by flow cytometry, whereas the NIP-specific Vaccibodies did not (Fig 3). To check for correct homodimerization, the Vaccibodies were metabolically labelled by growth of transfected cells in medium containing ^{35}S-methionine, vaccibodies were immunoprecipitated from supernatant using specific antibodies, and analyzed by SDS-PAGE. As would be expected from the theoretical consideration of Fig.2, both the $F_v{}^{NIP}$ $F_v{}^{315}$ and $F_{vI\text{-}E}$ $F_v{}^{315}$ transfectomas secreted dimeric Vaccibodies of ~ 130 kDa. After reduction of disulfide bonds, the Vaccibodies are degraded to monomeric chains of ~ 65 kDa (Fig. 20a).

Example 2

[0071] MHC class II-specific Vaccibodies enhance $\lambda2^{315}$ -specific stimulation of CD4+ T cells.

[0072]   Class II-specific and non-targeting NIP-specific Vaccibodies were mixed with antigen presenting cells (APC) and compared for their ability to induce specific T cell activation. Irradiated BALB/c splenocytes were used as APC. The BALB/c strain has the H-2^d haplotype, hence they express I-E^d molecules necessary for both targeting of the MHC II-specific Vaccibodies and presentation of the λ2^{315} epitope to specific CD4+ T cells.

[0073]   The APC were pulsed with the different Vaccibodies for 4h and subsequently washed. Washing was performed to reduce the chance that I-E^d – specific Vaccibodies in the culture medium could diminish T cell stimulation by blocking I-E^d molecules (Lunde, Western et al. 2002). Polarized Th2 cells from mice transgenic for a λ2^{315}-specific I-E^d restricted TCR (Lauritzen, Weiss et al. 1993) were added as responder T cells. The dose response curve in fig 6 shows that the λ2^{315} epitope was presented 100-1000 times more efficiently to TCR-transgenic Th2 cells when they were carried in the APC-targeted MHC II-specific Vaccibodies (both the ones with short and long human dimerization units) compared to the non-targeted NIP-specific Vaccibodies. It should be noted that Vaccibodies do not include an FcγR binding site; hence the NIP-specific Vaccibodies should not be able to enter cells via receptor-mediated endocytosis.

Example 3

[0074]   Level of anti-idiotypic antibodies in sera of mice that received Vaccibodies as proteins in saline s.c. in the absence of adjuvant.

[0075]   In the protein vaccination protocol, BALB/c mice were immunized twice, spaced two weeks apart, with 20 or 200µg MHC II- specific Vaccibodies or NIP-specific Vaccibodies in PBS. Note that no adjuvant was employed. Sera from immunized mice taken at various time points after the second vaccination were then analyzed for anti-idiotypic antibodies binding M315 in ELISA. The MHC II-specific Vaccibodies elicited significant higher anti-idiotypic antibody responses after 14 days after the second immunization than did NIP-specific Vaccibodies. Vaccibodies with a long human dimerization unit induced best anti-idiotypic Ab responses (Fig 7). Thus targeting of Vaccibodies enhanced anti-Id immuneresponses, however, by this route of immunization, also the non-targeted Vaccibodies induced some responses.

Example 4

[0076]   Protein Vaccibodies detected in serum after injection of DNA intramuscularly and in vivo electroporation.

[0077]   It has recently been described that skeletal muscle can produce antibodies after injection of Ig genes and electroporation (Tjelle 2004). We therefore investigated if functional Vaccibodies were produced by i.m. plasmid injection and electroporation. Since the F_V^{I-E} F_V^{315} Vaccibodies are specific for I-E^d molecules present in BALB/c (H-2d), these Vaccibodies should be rapidly absorbed by the I-E^d positive cells in BALB/c. By contrast, the non-targeted FvNIP F_V^{315} Vaccibodies should not be absorbed. Indeed, 14 days after a single injection of 50µg Vaccibody

plasmid in quadriceps and electroporation, F_v^{NIP} F_v^{315} Vaccibody protein was detected in significant amounts in serum, while there was no detectable F_v^{I-E} F_v^{315} (Fig 21a).

Example 5

[0078] Anti-Id antibodies in serum after injection of Vaccibody DNA intramuscularly and electroporation.

[0079] Analysis of the same day 14 sera samples for anti-idiotypic antibodies demonstrated that mice i.m. injected/electroporated with the MHC class II –targetd F_v^{I-E} F_v^{315} Vaccibody DNA, had developed antibodies that bound idiotypic Fv from the MOPC315.4 tumor (Fig 21b). This result was in distinct contrast to the lack of any anti-idiotypic antibody response in mice injected with the non-targeted F_v^{NIP} F_v^{315} Vaccibody DNA (Fig 21b). Taken together with the results described in example 4, the results demonstrate a formidable effect of targeting to MHC class II ($I-E^d$) positive cells for development of a strong humoral response. Control mice injected i.m. with 0.9% NaCl followed by electroporation had neither Vaccibodies nor anti-idiotypic Abs in day 14 sera (Fig 21a-c).

[0080] Example 6

[0081] Induction of protective immunity against the MOPC315.4 myeloma: Vaccibody DNA injection/electroporation.

[0082] Intramuscular vaccination with MHC class II-targeted F_v^{I-E} F_v^{315} Vaccibody plasmids and subsequent electroporation induced strong protection against a challenge with MOPC315.4 myeloma cells, $p<0.001$, compared to control mice injected with 0.9% NaCl and electroporated (Fig 23). By contrast, non-targeted F_v^{NIP} F_v^{315} Vaccibody plasmid immunization was ineffective compared to the saline control group, $p=0.2739$ (Fig 23). The appearance of tumor was delayed in mice vaccinated with F_v^{I-E} F_v^{315} compared to F_v^{NIP} F_v^{315} (Fig 22). One of the F_v^{I-E} F_v^{315} vaccinated mice developed a tumor of maximum 6mm (day 20) in diameter that regressed and was completely undetectable from day 28 (data not shown). The presence of M315 myeloma protein in sera confirmed the tumor size measurements (Fig 24). These results show that protection against the MOPC315.4 myeloma can be achieved by i.m. DNA vaccine followed by electroporation and that the protection requires targeting of the tumor-derived scFv to MHC class II (I-E^d) positive cells.

Example 7

[0083] Chemokines are functional as targeting units in the Vaccibody format

[0084] Supernatant from cells transfected with Vaccibody construct with MIP-1α in the targeting unit, long human dimerization unit and M315 scFv in the antigenic unit, were collected and tested in ELISA for binding to an anti-mouse MIP-1α mAb and 9A8 bio. The Vaccibodies containing MIP-1α bound to anti-MIP-1α mAb, while the NIP-specific Vaccibodies did not (Fig 25).

Example 8

[0085] The chemokine RANTES is functional as targeting unit in the Vaccibody format .

[0086] In the same manner, a vector with a gene encoding Vaccibodies like those described in example 7 was produced, with the exception that the targeting unit was the mouse chemokine RANTES. Supernatant from cells transfected with this construct was collected and tested in ELISA for the presence of Vaccibodies. The experiment showed that this Vaccibody variant was expressed and exported as a functional molecule.

Example 9

[0087] Flaggelin as targeting unit in the Vaccibody format.

[0088] In the same manner, a vector with a gene encoding Vaccibodies like those described in example 7 was produced, with the exception that the targeting unit was flaggelin. Supernatant from cells transfected with this construct will be collected and tested in ELISA for the presence of Vaccibodies.

Example 10

[0089] Soluble CD40 ligand as targeting unit in the Vaccibody format

[0090] In the same manner, a vector with a gene encoding Vaccibodies like those described in example 7 was produced, with the exception that the targeting unit was soluble CD 40 ligand from the mouse. Supernatant from cells

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transfected with this construct will be collected and tested in ELISA for the presence of Vaccibodies.

Example 11

[0091]   Anti-Toll-like-receptor 2 as targeting unit in the Vaccibody format.

[0092]   In the same manner, a vector with a gene encoding Vaccibodies like those described in example 7 was produced, with the exception that the targeting unit was a scFv with specificity for toll-like-receptor 2 from the mouse. Supernatant from cells transfected with this construct will be collected and tested in ELISA for the presence of Vaccibodies.

Example 12

[0093]   Anti-CD14 is functional as targeting units in the Vaccibody format

[0094]   In the same manner, a vector with a gene encoding Vaccibodies like those described in example 7 was produced, with the exception that the targeting unit was scFv with specificity for human CD 14. Supernatant from cells transfected with this construct was collected and tested in ELISA for the presence of Vaccibodies. The results showed that this Vaccibody variant was expressed and exported as a functional molecule.

Example 13

[0095]   Anti-HLA-DP is functional as targeting units in the Vaccibody format.

[0096] In the same manner, a vector with a gene encoding Vaccibodies like those described in example 7 was produced, with the exception that the targeting unit was scFv with specificity for HLA-DP. Supernatant from cells transfected with this construct was collected and tested in ELISA for the presence of Vaccibodies. The results showed that this Vaccibody variant was expressed and exported as a functional molecule.

Example 14

[0097] Tuberculosis antigen in the Vaccibody antigenic cassette.

[0098] A nucleic acid encoding a tuberculosis antigen (cattle antigen) will be inserted into the antigenic unit of the Vaccibody construct.

Example 15

[0099] Telomerase antigen in the Vaccibody antigenic cassette

[00100] hTERT, an antigenic region of the telomerase ribonucleoprotein, will be inserted into the antigenic unit of the Vaccibody construct.

Example 16

[00101] HIV Gp120 antigenic in the Vaccibody antigenic cassette

[00102] A nucleic acid encoding a gp120 derived molecule will be inserted into the antigenic unit of the Vaccibody construct.

Example 17

[00103] Vaccibodies with patient specific scFv of myeloma origin in the antigenic cassette.

[00104] This study has been initiated, but has not yet been completed.

[00105] Bone marrow aspirate from patients suffering from multiple myeloma can be collected. The mononuclear cells (MNC) can be separated using a density gradient solution of Ficoll-Isopaque (LymphoprepTM from Axis-Shield PoC AS). Total RNA can be isolated (TRIzol® Reagent from InvitrogenTM Life Technologies) from MNC, and cDNA can be made from mRNA (First-Strand cDNA Synthesis Kit from Amersham Biosciences (Not I-d(T)18 bifunctional primer)). This cDNA can be used as template in PCR with primers that amplify the V genes of the heavy or light chain of the multiple myeloma Ig. The sense primers are family specific and localized in the leader regions (VH1-7, VK1-6 and VL1-10), and the anti-sense primers are localized in the first part of the C regions (one primer each for IgG, IgA, kappa and lambda). PCR products can be ligated into a vector (pGEM®-T Easy Vector from Promega), and transformed into E.coli. DNA samples isolated from individual colonies can be sequenced. Getting the same sequence from three different colonies originating from three different PCRs confirms that the V regions from the myeloma Ig have been isolated. PCR SOEing can be performed and reamplification is done with primers including tags with sites for Sfil as described in Figure 19. For one patient such primers had the sequence:

5'TAVH

5' ACGTAGGCCTCGGTGGCCTGCAGATCACCTTGAAGGAGTCT

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3'TAVK

5'GATCCGGCCCTGCAGGCCTCATTTGATCTCCAGCTTGGTCCC

[00106] The resulting vector can be transiently transfected into 293E cells. Supernatants can be tested in ELISA for the presence of such Vaccibodies. They can also be injected into BALB/c mice. The presence of anti-Idiotypic antibodies can be measured in ELISAs against serum from the mice and serum from the patients.

[00107] All references cited herein are incorporated in their entireties by reference.

[00108] The description of the invention is merely exemplary in nature and, thus, variations that do not depart from the gist of the invention are intended to be within the scope of the invention. Such variations are not to be regarded as a departure from the spirit and scope of the invention.

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